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EphB4 Forward-Signaling Regulates Cardiac Progenitor Development in Mouse ES Cells

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ABSTRACT

Eph receptor (Eph)-ephrin signaling plays an important role in organ development and tissue regeneration. Bidirectional signaling of EphB4–ephrinB2 regulates cardiovascular development. To assess the role of EphB4–ephrinB2 signaling in cardiac lineage development, we utilized two GFP reporter systems in embryonic stem (ES) cells, in which the GFP transgenes were expressed in Nkx2.5^{+} cardiac progenitor cells and in α-MHC^{+} cardiomyocytes, respectively. We found that both EphB4 and ephrinB2 were expressed in Nkx2.5-GFP^{+} cardiac progenitor cells, but not in α-MHC-GFP^{+} cardiomyocytes during cardiac lineage differentiation of ES cells. An antagonist of EphB4, TNYL-RAW peptides, that block the binding of EphB4 and ephrinB2, impaired cardiac lineage development in ES cells. Inhibition of EphB4–ephrinB2 signaling at different time points during ES cell differentiation demonstrated that the interaction of EphB4 and ephrinB2 was required for the early stage of cardiac lineage development. Forced expression of human full-length EphB4 or intracellular domain-truncated EphB4 in EphB4-null ES cells was established to investigate the role of EphB4-forward signaling in ES cells. Interestingly, while full-length EphB4 was able to restore the cardiac lineage development in EphB4-null ES cells, the truncated EphB4 that lacks the intracellular domain of tyrosine kinase and PDZ motif failed to rescue the defect of cardiomyocyte development, suggesting that EphB4 intracellular domain is essential for the development of cardiomyocytes. Our study provides evidence that receptor-kinase-dependent EphB4-forward signaling plays a crucial role in the development of cardiac progenitor cells. J. Cell. Biochem. 116: 467–475, 2015. © 2014 The Authors. *Journal of Cellular Biochemistry* published by Wiley Periodicals, Inc.

KEY WORDS: EMBRYONIC STEM (ES) CELLS; CARDIOMYOCYTES; EphB4; ephrinB2; CARDIAC PROGENITOR CELLS; Nkx 2.5; α-MHC

Understanding the molecular and cellular mechanisms underlying stem cell differentiation into cardiomyocytes will provide insights into therapeutic applications for prevention and treatment of heart failure. A strong contender involved in stem cell differentiation is Eph-ephrin signaling. Fourteen Eph receptor tyrosine kinases are catalogued into EphA and EphB subclasses based on their affinity for ephrin ligands that are either glycosylphosphatidylinositol (GPI)-linked (ephrinA) or transmembrane (ephrinB) proteins [Committee, 1997]. Eph-ephrin signaling plays important roles in a variety of processes during embryonic development, including the targeting behavior of migratory neurons, vascular cell assembly, and angiogenesis

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Kang Chen and Hao Bai equally contribute to this work.

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[Gale and Yancopoulos, 1999; Poliakov et al., 2004; Egea and Klein, 2007; Arvanitis and Davy, 2008; Pasquale, 2008]. Rather than long range communication, Eph receptors and their ligands signal at restricted sites of direct cell–cell contact, resulting in reciprocal bidirectional events between interacting cells [Davis et al., 1994; Bruckner and Klein, 1998; Gale and Yancopoulos, 1999; Poliakov et al., 2004; Egea and Klein, 2007; Arvanitis and Davy, 2008; Pasquale, 2008]. When EphB4 receptor interacts with ephrinB2 ligand, the EphB4-forward signaling exerts in a receptor-kinase-dependent manner, and ephrinB2-reverse signaling is independent of the tyrosine kinase of EphB4 receptor [Fuller et al., 2003; Chrencik et al., 2006].

The potential importance of EphB4–ephrinB2 signaling in cardiovascular development has been demonstrated by loss-of-function approaches [Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999; Gerety and Anderson, 2002; Cowan et al., 2004]. During embryonic development, EphB4 and ephrinB2 are expressed in the vascular endothelium and in the heart ventricles [Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999; Gerety and Anderson, 2002; Cowan et al., 2004]. Global knockout of EphB4 or ephrinB2 in mice results in not only defective vascular development, but also arrested heart development, including decrease of heart size, incompletion of cardiac looping, failure of endocardium expansion, failure of myocardial trabeculation, and thickened cardiac valves [Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999; Gerety and Anderson, 2002; Cowan et al., 2004]. Knockout of EphB4 and the cognate ligand ephrinB2 is embryonic lethal in mice and therefore its role in cardiac lineage development remains poorly defined.

Pluripotent stem cells, such as embryonic stem (ES) cells and induced-pluripotent stem (iPS) cells, provide an excellent model system for investigation of molecular and cellular mechanisms of cardiac development and cardiac diseases [Chen et al., 2008]. Our previous studies of ES cells demonstrated that endothelial cells provide a stem cell niche to promote ES cell differentiation into cardiomyocytes, and that EphB4 signaling regulates endothelial niche function [Chen et al., 2010]. In the current study, we found that EphB4 and ephrinB2 were expressed in Nkx2.5^{+} cardiac progenitor cells, but not in α-MHC^{+} cardiomyocytes during murine ES cell differentiation. Disrupting the interaction of EphB4 and ephrinB2 at the early stage of ES cell differentiation impaired cardiac lineage development. Reconstitution of EphB4 in EphB4-null ES cells demonstrated that EphB4 intracellular domain was essential for ES cell differentiation to cardiomyocytes. Our data indicates that EphB4-forward signaling is involved in cardiac progenitor development.

MATERIALS AND METHODS

CELL CULTURE

The α-MHC-GFP mouse ES cell line (CGR8-GFP) was generously provided by Dr. Richard T. Lee (Harvard Medical School, Boston, MA). The expression of enhanced GFP (EGFP) transgene is under the control of cardiac muscle specific α−myosin heavy chain (α−MHC) [Takahashi et al., 2003]. Nkx2.5-GFP mouse ES cell line (Nkx2.5-EmGFP) was generously provided by Dr. Edward Hsiao (Gladstone Institute of Cardiovascular Disease). The emerald GFP (EmGFP) reporter is knock-in at Nkx2.5 locus 26 amino acids downstream of the native ATG site in a human BAC vector [Hsiao et al., 2008].

Mouse ES cells were cultured as we previously described [Wang et al., 2004; Chen et al., 2010]. Briefly, ES cells were maintained on a mouse feeder cell line (SNL) in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acid, 150 μM monothioglycerol (MTG;Sigma–Aldrich, St. Louis, MO), 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 ng/ml leukemia inhibitory factor (LIF; Chemicon International). The ES cells were cultured on gelatin-coated plates for two passages to remove feeder cells before EB differentiation.

CARDIOMYOCYTE DIFFERENTIATION OF ES CELLS

ES cell differentiation into embryoid bodies (EBs) was performed as described previously [Chen et al., 2010]. Briefly, 400 ES cells were suspended in hanging-drop cultures in 30 μl differentiation medium containing Iscove modified Dulbecco's medium (IMDM), 15% defined-FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. After 5 days of differentiation, individual EBs were transferred to a gelatin-coated well in 48-well plates. Differentiation medium was changed every 3 days. After 5 to 8 days of differentiation, GFP^{+} cardiomyocytes from α−MHC−GFP, and GFP^{+} cardiac progenitor cells from Nkx2.5-GFP ES cells were observed under a fluorescent microscope.

ES cell differentiation was carried out with or without TNYL-RAW (5 μM). The TNYL-RAW peptide (TNYLFSPNGPIARAW) is an antagonist of EphB4–phrinB2 binding to EphB4 [Koolpe et al., 2005]. The control peptide (WYPSNTRPGILNAFA) contains a scramble sequence of TNYL-RAW. Both the TNYL-RAW and control peptide were synthesized by EZBiolab (www.ezbiolab.com; Westfield, IN). TNYL-RAW, or a control peptide was added into the differentiation medium at different time points, depending on experiment design.

QUANTITATIVE PCR (Q-PCR) ANALYSIS

Undifferentiated ES cells and differentiated EBs were harvested at different time points. Total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). One microgram RNA was used to generate cDNA in 20 μl by reverse transcription (RT) using SuperScript III reverse transcriptase (Invitrogen). For quantitative PCR (real-time PCR), 0.5 μl of cDNA was added to 19.5 μl of master mix containing 1X SYBR Green (Bio-Rad Laboratories, Hercules, CA) and 25 mM sense and antisense primers. Triplicates of the cDNA were amplified for 40 cycles with the iCycler iQ real-time thermal cycler (Bio-Rad Laboratories). The PCR product level was expressed as Ct, the amplification cycle at which the emission intensity of the product rises above an arbitrary threshold level. Specific primers used for Q-PCR are as previously described [Chen et al., 2010].

FLOW CYTOMETRIC ANALYSIS

Differentiated EBs at different time points were trypsinized for 2 min, dissociated with gentle agitation and passed through a 30 μm pre-

separation filter (Miltenyi Biotec Inc., Auburn, CA) to produce a single cell suspension. Cells (1×10^6) were then stained with primary antibodies as follows: EphB4 at 1:100 dilution (R&D Systems, Minneapolis, MN), ephrinB2 at 1:100 dilution (R&D Systems), and rat IgG2a (control antibodies) at 1:100 dilution. PE- or APC-conjugated secondary antibodies were used for flow cytometric analysis. Dead cells were gated out by a DNA dye (7-Amino-Actinomycin D, 7AAD staining). Isotype-matched control antibodies were used to determine the background staining. All antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). The cells were analyzed on FACSCalibur (BD Biosciences) with CellQuest software. Data analysis was performed using CellQuest or FlowJo Software.

Cell sorting was performed using Fluorescence Activated Cell Sorting (FACS) on FACSAria II (BD Biosciences).

GENERATION OF KO-HB4 CELLS BY LENTIVIRAL TRANSDUCTION

The human EphB4 gene was cloned into a lentiviral vector pLentiGFPtc, in which EphB4 expression was driven by a mini-CMV inducible promoter, and constitutive expression of fluorescence marker GFP was driven by an individual EF-1alpha promoter [Bai et al., 2012]. The lentiviral vector pLentiGFPtc–EphB4 was transfected into 293 T-cells, respectively for lentivirus preparation. The lentivirus was concentrated by PEG-8000 and applied to transduce the mouse ESCs, as previously described [Wang et al., 2007; Bai et al., 2012]. Using fluorescence microscopy, the GFP$^+$ mouse ES cell colonies were manually picked up. After five passages of selection, the mouse ES cells capable of induced expression of EphB4 (KO-hB4) were established.

WESTERN BLOT ANALYSIS

Expression of Ephb4 was monitored by Western blot analysis. To induce EphB4 expression, doxycycline of various concentrations (0, 500, and 1,000 ng/ml) was added to the mouse ES cell growth medium for 2 days, and then the cells were lysed in RIPA buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 μM leupeptin, 150 mM NaCl, 50 mM Tris, pH 7.4) supplemented with 1% protease inhibitor cocktail (Sigma–Aldrich) [Bai et al., 2012]. Western blot analyses were performed with anti-EphB4 antibodies (BD Biosciences) as primary antibodies and anti-rabbit IgG-HRP antibodies (Sigma–Aldrich) as secondary antibodies. The protein expression levels were quantified using Photoshop software based on band area and gray scale.

RESULTS

EXPRESSION OF EphB4 AND ephrinB2 IN NKX2.5$^+$ CARDIAC PROGENITOR CELLS, BUT NOT IN α-MHC$^+$ CARDIOMYOCYTES

To investigate how EphB4 signaling regulates cardiomyocyte generation, we assessed EphB4 and ephrinB2 expression during ES cell differentiation to cardiomyocytes. We induced ES cell differentiation by hanging-drop method to form uniformed EBs (Fig. 1A). After 5 days in hanging-drop, individual EBs were transferred to a gelatin-coated well in 48-well plates, in which attached EBs undergo cardiomyocyte development [Chen et al., 2010]. The GFP$^+$ cardiomyocytes from α-MHC-GFP mouse ES cells usually form cell clusters that generate spontaneously beating areas after 8 to 9 days of differentiation (Fig. 1B). The spontaneous beating EBs that contained cardiomyocytes were scored at different days.

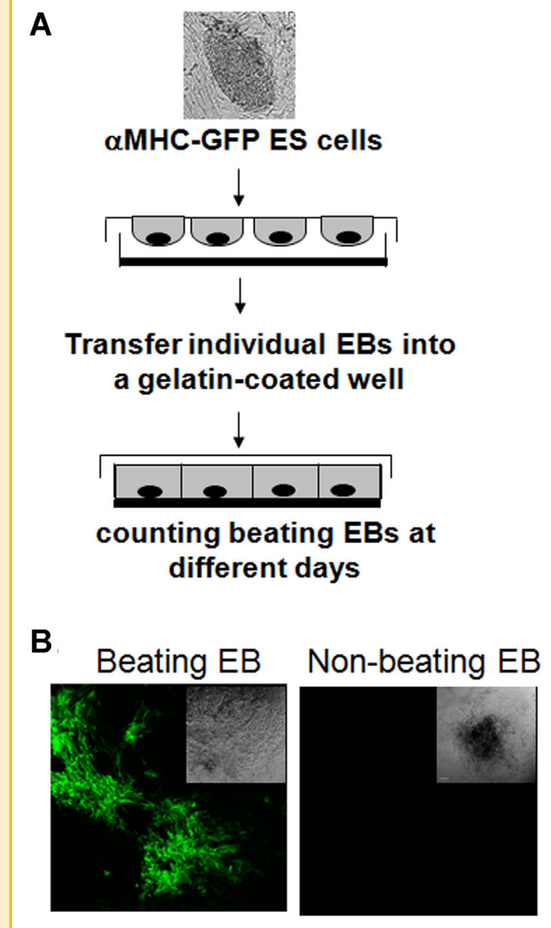

Fig. 1. ES cell differentiation into beating embryoid bodies (EBs) containing cardiomyocytes. (A) Schematic diagram of ES cell differentiation. EBs were initialized in hanging drops. Four hundreds of undifferentiated ES cells were used to form each EB. Individual EBs were transferred to gelatin-coated wells at day 5. Spontaneous contracting EBs (beating EBs) were observed after EB attachment. (B) α-MHC-GFP ES cells were differentiated into EBs. GFP expressing cells and beating EBs were analyzed under a fluorescent microscope at different time points. Photos represented a beating EB and non-beating at day 10.

Two ES cell lines were used in cardiomyocyte differentiation in our study: (i) Nkx2.5-GFP ES cells in which GFP expression is controlled by Nkx2.5 promoter that is activated in cardiac progenitors [Hsiao et al., 2008]; and (ii) α-MHC-GFP ES cells in which GFP expression is controlled by the α-cardiac MHC promoter to ensure exclusive expression in spontaneous contracting cardiomyocytes in beating EBs [Takahashi et al., 2003]. We determined the kinetics of GFP expression in Nkx2.5-GFP ES cells and in α-MHC-ES cells during ES cell differentiation under a fluorescent microscope at different time points. In our differentiation condition, GFP expression in Nkx2.5-GFP ES cells emerged at day 6 (Fig. 2A) and reached to the highest levels at day

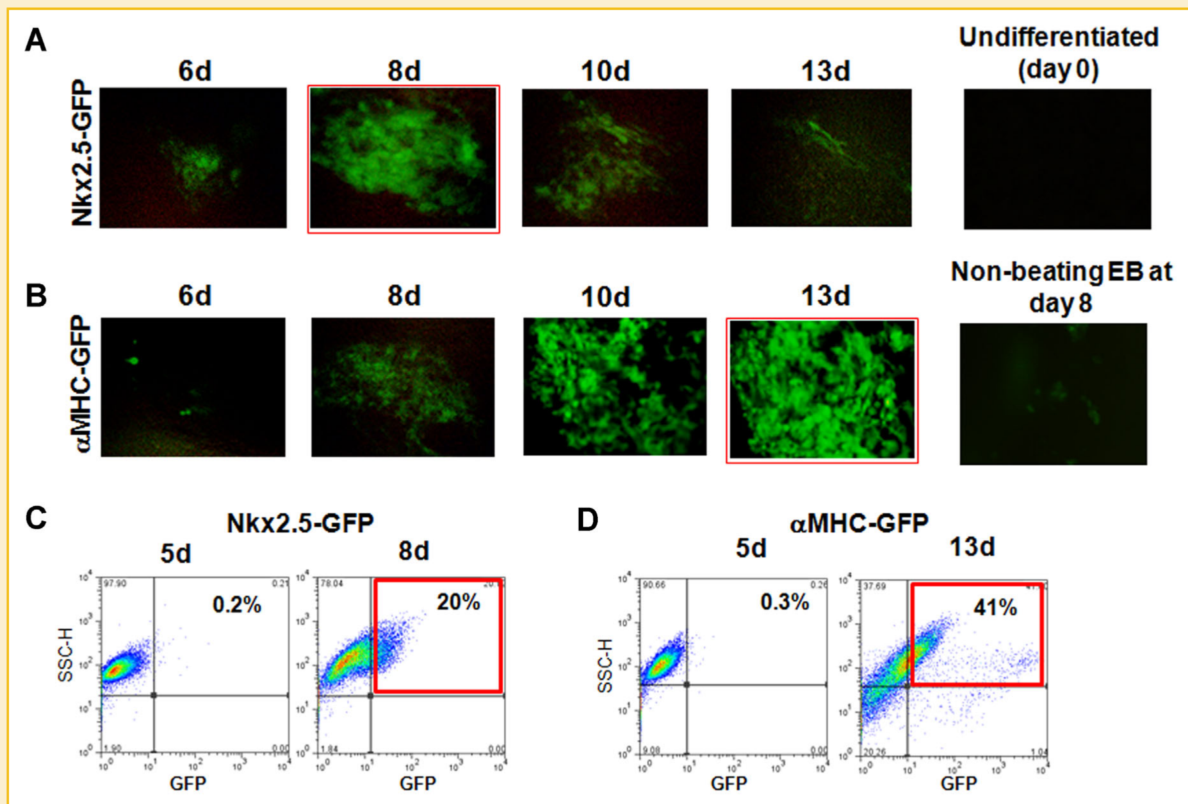

Fig. 2. Cardiac differentiation of Nkx2.5-GFP ES cells and α-MHC-GFP ES cells. (A) and (B) Nkx2.5-GFP ES cells and α-MHC-GFP ES cells were induced for cardiac differentiation. GFP expression from both ES cell lines were photographed from day 6 to 13, as indicated. Whereas GFP expression of Nkx2.5-GFP ES cells peaked at day 8, GFP expression of α-MHC-GFP ES cells reached to the highest levels at day 13. (C) and (D) Flow cytometric analysis of Nkx2.5-GFP ES cells and α-MHC-GFP ES cells at day 8 and day 13, respectively. Cells from both Nkx2.5-GFP ES cells and α-MHC-GFP ES cells at day 5 were used as a control that showed GFP^+ cells less than 0.5%. Data are representative from three independent experiments.

8, whereas GFP expression in α-MHC-GFP ES cells reached the highest levels at day 13 (Fig. 2B). Flow cytometry indicated that approximately 20% of the differentiated cells in EBs were GFP-positive in Nkx2.5-GFP ES cells at day 8 (Fig. 2C), whereas approximately 40% of the differentiated cells in EBs were GFP-positive in α-MHC-GFP ES cells at day 13 (Fig. 2D). To evaluate gene expression profiles in cardiac progenitors and in cardiomyocytes, we used FACS to sort GFP^+ cells from Nkx2.5-GFP ES cells at day 8 and GFP^+ cardiomyocytes from α−MHC-GFP ES cells at day 13, respectively. Real-time PCR analysis of sorted GFP^+ cells indicated that Flk1, the mesoderm progenitor marker (Fig. 3A), and Nkx2.5, cardiac progenitor marker (Fig. 3B) were expressed in high levels in $Nkx2.5^+$ cardiac progenitors, compared to their expression in $\alpha\text{-MHC}^+$ cardiomyocytes. Meanwhile, GATA4, a critical transcription factor for cardiac development, was expressed in both cardiac progenitors and cardiomyocytes (Fig. 3C). Interestingly, both EphB4 and ephrinB2 were expressed in $Nkx2.5^+$ cardiac progenitors, but not in $\alpha\text{-MHC}^+$ cardiomyocytes (Fig. 3D and E), suggesting a role of EphB4–ephrinB2 signaling in early rather than later stage of cardiac lineage development.

BLOCKAGE OF THE INTERACTION OF EphB4 AND ephrinB2 IN AN EARLY STAGE OF ES DIFFERENTIATION INHIBITS CARDIOMYOCYTE GENERATION

To examine whether the interaction of EphB4 and ephrinB2 contributes to cardiomyocyte generation from ES cells, we used TNYL-RAW peptide to block the interaction of EphB4 and ephrinB2 during α-MHC-GFP ES cell differentiation. As shown in Figure 4A and B, the frequency of beating EBs and the number of GFP^+ cardiomyocytes were significantly decreased in the presence of TNYL-RAW peptide during cardiomyocyte differentiation, suggesting that a binding of EphB4 and ephrinB2 is required for cardiomyocyte generation. To further analyze the effect of EphB4–ephrinB2 signaling on cardiomyocyte generation, TNYL-RAW peptide was added at different time points during ES cell differentiation. As we expected, the effective time of EphB4–ephrinB2 signaling was in an early developmental stage. The addition of TNYL-RAW at day 0 (EB0) or day 2 (EB2) during ES cell differentiation dramatically decreased the numbers of beating EBs, whereas the addition of TNYL-RAW at day 5 (EB5) or later had no effect on the development of beating EBs (Fig. 4C), suggesting an important role of EphB4–ephrinB2 interaction/signaling during the early phase of cardiac lineage development.

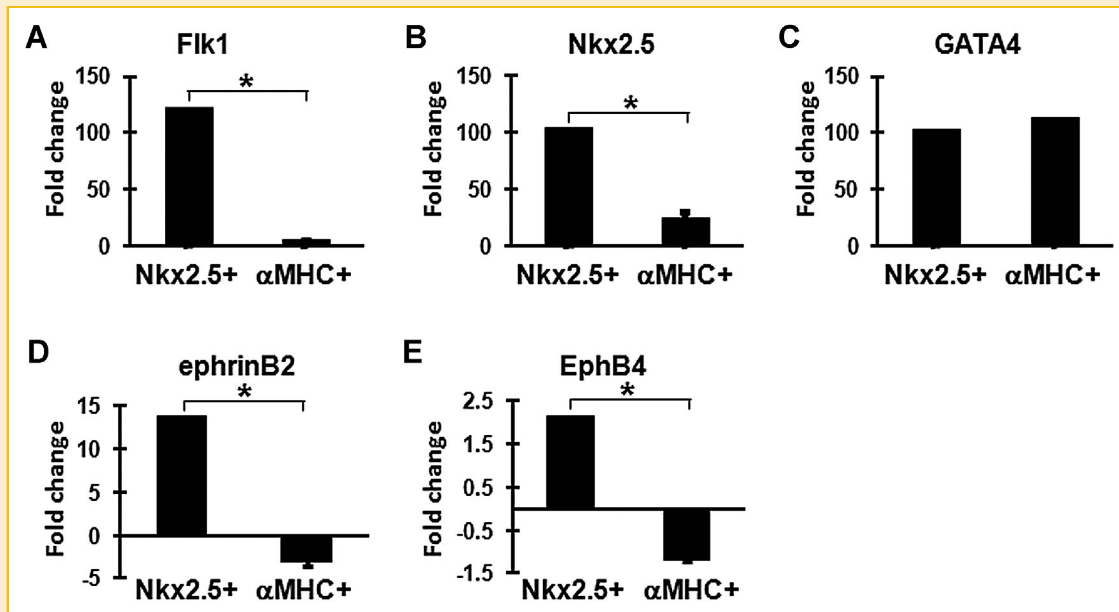

Fig. 3. Quantitative analysis of gene expression by real-time PCR. GFP^+ cells at day-8 EBs from Nkx2.5-GFP ES cells ($Nkx2.5^+$), and GFP^+ cells at day-13 EBs from α-MHC-GFP ES cells (αMHC^+) were sorted by FACS. Total RNA was extracted from sorted GFP^+ cells with TRIzol reagent, and subject to real-time PCR analysis for the gene expression of Flk1 (A), Nkx2.5 (B), GATA4 (C), ephrinB2 (D), and EphB4 (E). Undifferentiated ES cells (ESC) were used as a control. GAPDH was used as a standard. Error bars represent standard deviation and * represents $P < 0.05$.

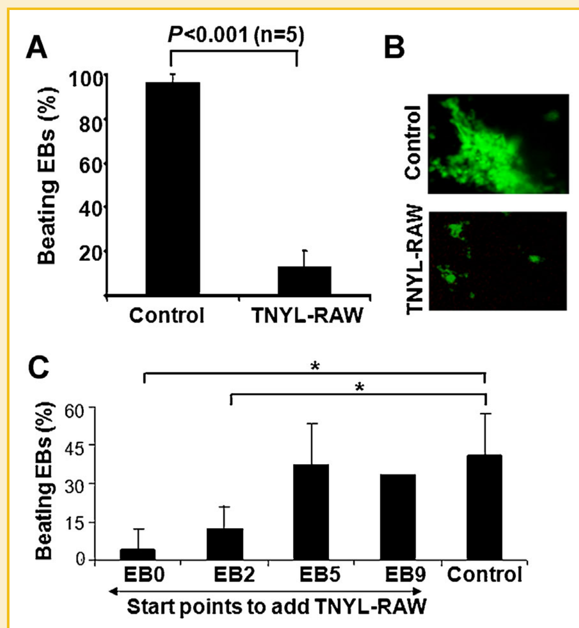

Fig. 4. Impairing cardiac differentiation by blocking the interaction of EphB4 and ephrinB2 with TNYL-RAW peptide. α-MHC-GFP ES cells were differentiated into beating EBs in the presence of TNYL-RAW peptide (TNYLFSPNGPIARAW). The peptides were added at day 0, and through the end of differentiation. The frequency of beating EBs (A) and GFP^+ cells (B) were analyzed at day 12. (C) The addition of TNYL-RAW peptides was initialized at different time points during ES cell differentiation: day 0 (EB0), day 2 (EB2), day 5 (EB5), or day 9 (EB9). ES cell differentiation was compared in the presence of the control peptide as added starting at day 0. Data are representative from three independent experiments. Error bars represent standard deviation and * represents $P < 0.05$.

EphB4 FORWARD-SIGNALING IS CRUCIAL FOR CARDIOMYOCYTE GENERATION

We previously demonstrated that the differentiation of ES cells into cardiomyocytes is impaired in EphB4-null ES cells [Wang et al., 2004], and this defect of cardiomyocyte generation in the absence of EphB4 expression can be rescued by co-culture of normal endothelial cells [Chen et al., 2010]. One possible explanation to this observation is that endothelial cells provide EphB4-mediated function to facilitate cardiomyocyte differentiation in EphB4-null ES cells. To analyze the function of EphB4 in promoting cardiomyocyte generation, we established an EphB4-expressing reconstitution system by a lentiviral system [Lois et al., 2002] to ectopically express the full-length of human EphB4 gene (hB4) in EphB4-null ES cells (KO). The forced expression of human EphB4 in EphB4-null ES cells (KO-hB4) was confirmed by real-time PCR analysis (Fig. 5A), Western blotting (Fig. 5B) and flow cytometry (Fig. 5C). As shown in Figure 5D, forced expression of human EphB4 in EphB4-null ES cells (KO-hB4) fully restored EB beating activity, confirming a critical role of EphB4 in cardiomyocyte generation.

In a further test, we used the inducible lentiviral system to express full length human EphB4 gene or a truncated EphB4 gene that lacks the intracellular domain in EphB4-null ES cells (Fig. 6A and C). The addition of 500 ng/ml of doxycycline in culture medium was sufficient to induce ectopic expression (Fig. 6B), as in our previous study [Bai et al., 2012]. Forced expression of full-length EphB4 in EphB4-null ES cells (KO-hB4) restored the development of beating-EBs, and had similar kinetics to wild-type ES cells (control) (Fig.6D). However, forced expression of the truncated EphB4 gene in EphB4-null ES cells (KO-ΔhB4) failed to rescue the defect of cardiomyocyte

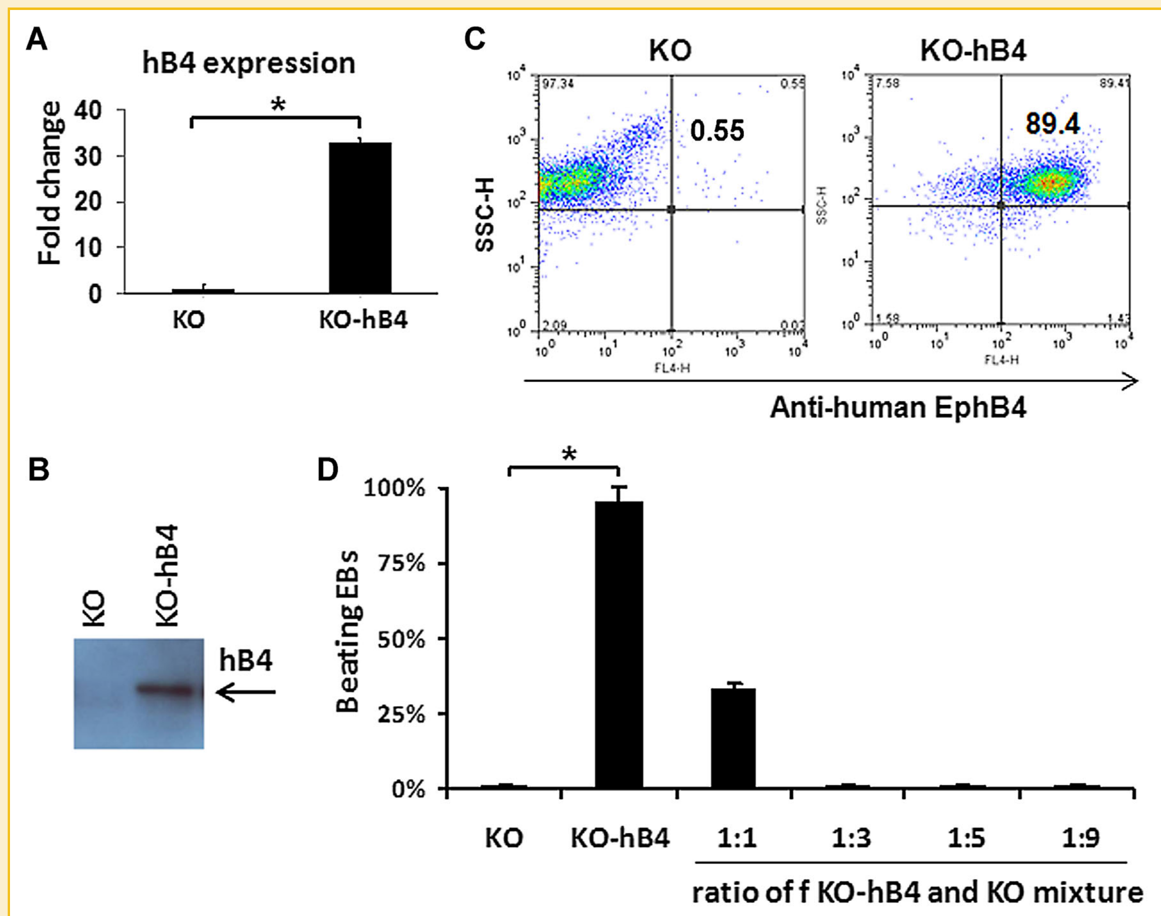

Fig. 5. Rescue of cardiomyocyte-defect in EphB4-null ES cells by ectopic expression of human EphB4 gene. The full-length of human EphB4 cDNA was used to express human EphB4 gene in EphB4-null ES cells (KO) by a lentiviral vector to establish ES cell line expressing human EphB4 (KO-hB4). The expression of EphB4 was analyzed by real-time PCR (A), Western blotting (B), and flow cytometry using anti-human EphB4 antibodies (C). (D) Analysis of beating EBs. ES cells of KO, KO-hB4, and mixtures of KO-hB4 ES cells with KO ES cells at different ratios (1:1, 1:3, 1:5, and 1:9) were induced for cardiomyocyte differentiation. The frequency of beating EBs was determined at day 12. Data are representative from three independent experiments. Error bars represent standard deviation and * represents $P < 0.05$.

generation (Fig. 6D), indicating that EphB4-forward signaling is required for cardiomyocyte generation in ES cells.

To gain more insight into the mechanism of EphB4 function, we tested whether EphB4-expressing ES cells are able to provide paracrine signal for neighboring cells, and thereby rescue the cardiac defect in EphB4-null ES cells. We mixed KO-hB4 ES cells and KO ES cells at different ratios, and then subjected the mixture to cardiomyocyte differentiation. When KO-hB4 cells and KO cells were mixed at 1:1 ratio, approximately 33% of the EBs contained a beating activity. When the ratio of KO-hB4 cells versus KO cells was reduced to 1:3, beating-EBs decreased to less than 1% (Fig. 5D). These data demonstrated that, at least in tested expression level, EphB4 expression in neighboring cells were insufficient to promote cardiomyocyte generation in EphB4-null ES cells.

DISCUSSION

It is well-known that the receptor-tyrosine kinase, EphB4, and its ligand, ephrinB2, play a crucial role in angiogenesis. During vascular development, EphB4 and ephrinB2 are specifically expressed in venous and arterial endothelial cells, respectively [Wang et al., 1998; Gale et al., 2001; Shin et al., 2001]. In addition to the vascular defects, phenotypes of arrested heart development, including decreased heart size, incompletion of cardiac looping, failure of endocardium expansion, failure of myocardial trabeculation, and thickened cardiac valves, have been observed in EphB4 or ephrinB2 gene knockout mice [Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999; Gerety and Anderson, 2002; Cowan et al., 2004]. We previously demonstrated that EphB4 knockout in ES cells results in the defect of cardiomyocyte generation [Wang et al., 2004]. However, EphB4 is expressed in endothelial cells underneath contracting cardiomyocytes, but not in mature cardiomyocytes [Chen et al., 2010]. In this study, we found that in the absence of EphB4, the generation of beating cardiomyocytes were significantly reduced in EphB4-null ES cells (Fig. 6D), consistent with our previous study [Chen et al., 2010]. However, neither EphB4 nor ephrinB2 was expressed in cardiomyocytes (α-MHC-GFP ES cells) (Fig. 3D and E), suggesting that EphB4 signaling is not involved in the later stage of cardiomyocyte generation. Although EphB4 is not

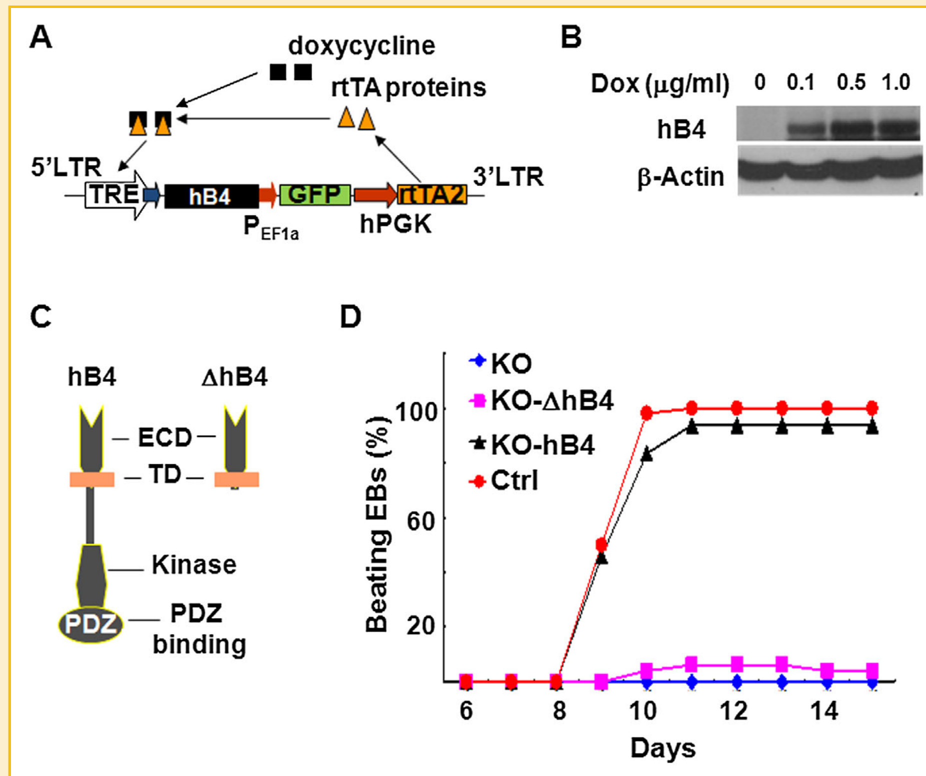

Fig. 6. Requirement of EphB4-forward signaling in cardiac differentiation of ES cells. (A) Schematic diagram of an inducible lentiviral system. (B) Western blotting analysis human EphB4 expression induced by doxycycline (Dox) at different concentrations. (C) Schematic diagram of human EphB4 protein with a structure of truncated intracellular domain, including kinase domain and PDZ domain (ΔhB4). (D) Analysis of beating EBs. EphB4-null ES cells (KO), EphB4-null ES cells expressing human EphB4 (KO-hB4), EphB4-null ES cells expressing truncated EphB4 (KO-ΔhB4), and wild-type ES cells (Ctrl) were differentiated to cardiomyocytes. The frequency of beating EBs was analyzed at different time points.

expressed in cardiomyocytes, the function of EphB4 in regulating embryonic cardiac development can be accomplished by endothelial cells underneath cardiomyocytes [Chen et al., 2010].

ES cell-derived cardiomyocytes express cardiac gene products in a developmentally controlled manner. In early myocardial development, mRNAs encoding GATA4 and Nkx2.5 transcription factors appear in EBs before mRNAs encoding atrial natriuretic factor (ANF), myosin light chain (MLC)-2v, α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), Na^{+}-Ca^{2+} exchanger, and phospholamban [Boheler et al., 2002]. Nkx2.5, the homeobox gene, is the known earliest marker of the cardiac lineage in vertebrate embryos. Nkx2.5 expression is first detected in mesodermal cells specified to form the heart at embryonic day 7.5 in the mouse, and its expression is maintained throughout the developing and adult heart [Lien et al., 1999]. Nkx2.5 expression has been used to identify cardiac progenitors in differentiated ES cells [Hidaka et al., 2003]. In contrast, α-MHC is among cardiac-specific genes detectable relatively late in the developing heart and is the specific marker for cardiomyocytes [Gulick et al., 1991; Morkin 2000; Boheler et al., 2002; Takahashi et al., 2003]. Therefore, Nkx2.5 and α-MHC were used as early and later markers of cardiac lineage in our study.

By analysis of sorted GFP^{+} cells, we found that EphB4 and ephrinB2 were expressed in $Nkx2.5^{+}$ cardiac progenitor cells, but not in α-MHC^{+} cardiomyocytes (Fig. 3). Interestingly, $Nkx2.5^{+}$ cells also highly expressed Flk1 (Fig. 3A), a marker to identify cardiac stem/progenitor cells in ES cells [Sitnicka et al., 2003; Larochelle et al., 2011]. To test whether EphB4–ephrinB2 signaling is involved in early stage of cardiac lineage development, we utilized the EphB4/ephrinB2 antagonist peptide that binds to the ephrin-binding pocket of EphB4 with a high affinity at nanomolar range [Chrencik et al., 2006; Noberini et al., 2011]. TNYL-RAW peptide specifically binds to the ephrin-binding pocket of EphB4, but not to EphB2 or EphB3, thus selectively blocks EphB4 receptor activation by ephrinB2 [Koolpe et al., 2005; Chrencik et al., 2006; Salvucci et al., 2006; Noberini et al., 2011; Xiong et al., 2011; Zhang et al., 2011]. By interfering EphB4–ephrinB2 interaction at different time points during ES cell differentiation to cardiomyocytes, we demonstrated that EphB4–ephrinB2 signaling was required in an early stage of cardiac lineage development (Fig. 4).

Over the past decade, essential roles for Eph receptor-tyrosine kinases and their ligands, the ephrins, emerged from studies of embryonic development [Frisen et al., 1999]. EphB4–ephrinB2 interaction elicits bidirectional signaling events upon cell–cell

contact [Frisen et al., 1999; Kullander and Klein, 2002]. Bidirectional signaling of EphB4 and ephrinB2 is propagated not only downstream of EphB4 (forward-signaling) but also downstream of ephrinB2 (reverse-signaling). The EphB4-forward signaling exerts in a receptor-kinase-dependent manner, and ephrinB2-reverse signaling is through an EphB4-receptor-kinase-independent mechanism. It has been reported that between 8.0 and 9.5 days post coitum (dpc) of mouse embryos, EphB4 is expressed in myocardial cells, but not in endocardial cells. After 9.5 dpc, EphB4 expression is decreased, suggesting a role of EphB4 in development of the primitive heart [Ruiz et al., 1994]. Our data demonstrated for the first time that EphB4 and ephrinB2 are expressed in cardiac progenitors. Interestingly, ectopic expression of full-length EphB4 is sufficient to restore beating-EB development in EphB4-null ES cells, whereas truncated EphB4 failed to rescue the defect of cardiomyocyte generation (Fig. 6), suggesting that EphB4 kinase and downstream signaling were critical for cardiomyocyte development. However, considering our previous observation that co-culture of endothelial cells are able to rescue the defect of cardiomyocyte differentiation of EphB4-null ES cells [Chen et al., 2010], the role of EphB4 kinase and downstream signaling in cardiomyocyte differentiation may be carried out in an indirect mechanism. Further experiments are required to reveal the complex network of EphB4–ephrinB2 signaling in cardiomyocyte differentiation related events.

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